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ENTRY INTO HOST CELLS BY *LEGIONELLA*

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1. ABSTRACT

Many respiratory diseases are caused by extracellular bacterial pathogens; however, two very important lung infections are due to intracellular pathogens, Legionnaires' disease and tuberculosis. Legionnaires' disease remains problematic due to our inability to predict where sporadic epidemics will occur and the speed at which the bacterium debilitates its victims. The development of better methods for prevention would greatly alleviate public concern and the economic impacts of eradication efforts where infections occur. *Legionella*, the causative agent of Legionnaires' disease, has been shown to replicate within eukaryotic cells both during disease and in the environment. During disease these bacteria are found primarily within macrophages, though they have the ability to enter and survive within a number of different mammalian cell types. In the environment *Legionella* replicate within free-living protozoa. Thus, the ability to enter into host cells successfully and efficiently is critical to the ability of *Legionella* to survive. The process by which *Legionella* gains access to the intracellular environment involves a number of steps; including, finding an appropriate host cell, adherence, signal transduction, entry and initial survival. Unless *Legionella* accomplishes each of these steps properly, few viable bacteria will be observed intracellularly and reduced intracellular replication may occur. However, the importance of each of these individual steps in the pathogenesis of *Legionella* is unclear. Herein we discuss the potential mechanisms of entry by *Legionella* into host cells, a critical early event in the production of Legionnaires' disease.

2. INTRODUCTION

Sporadic epidemics of Legionnaires' disease have continued to plague technologically advanced countries despite our identification of the infecting organism and many environmental reservoirs. *Legionella* is the causative agent of Legionnaires' disease, named for the 29 Legionnaires killed by a mysterious pneumonia at an American Legion convention in Philadelphia during July of 1976 (1, 2). Although a large number of *Legionella* species have been identified and many of these have been shown to cause disease in humans, *L. pneumophila* is responsible for approximately 90% of the pneumonias attributed to these bacteria (3-5). For this reason, the majority of the research into causes of Legionnaires' disease has been carried out on *L. pneumophila*. *Legionella* spp. are ubiquitous in the environment, replicating intracellularly in amoebae and other protozoa (6, 7). Rowbotham first described the ability of *L. pneumophila* to infect *Acanthamoeba* and *Naegleria* spp. (8). Subsequent studies have shown that *L. pneumophila* can replicate within fourteen species of amoebae and two species of ciliated protozoa (6). When located intracellularly in amoebae, *L. pneumophila* can avoid contact with chemicals (9) and biocides (10) used to purify water. Growth in amoebae has also been shown to confer resistance of *L. pneumophila* to antibiotics (11), biocides (11) and oxidizing agents (12). *L. pneumophila* may also persist inside amoebal cysts, which are even more resistant to harsh environmental conditions (10, 13).

The ability of *L. pneumophila* to parasitize protozoa and macrophages indicates that these bacteria have developed strategies to circumvent bactericidal mechanisms of host cells. Since *L. pneumophila* replicates primarily in macrophages during disease, its pathogenicity for humans is dependent on the outcome of the interaction between the bacterium and professional phagocytes it encounters in the host. The pathogenic mechanisms used by intracellular respiratory pathogens are not well understood. A variety of mechanisms have been implicated in the intracellular survival of pathogenic bacteria. Mechanisms commonly used include the ability to exit the vacuole gaining access to the cytoplasm, resistance to

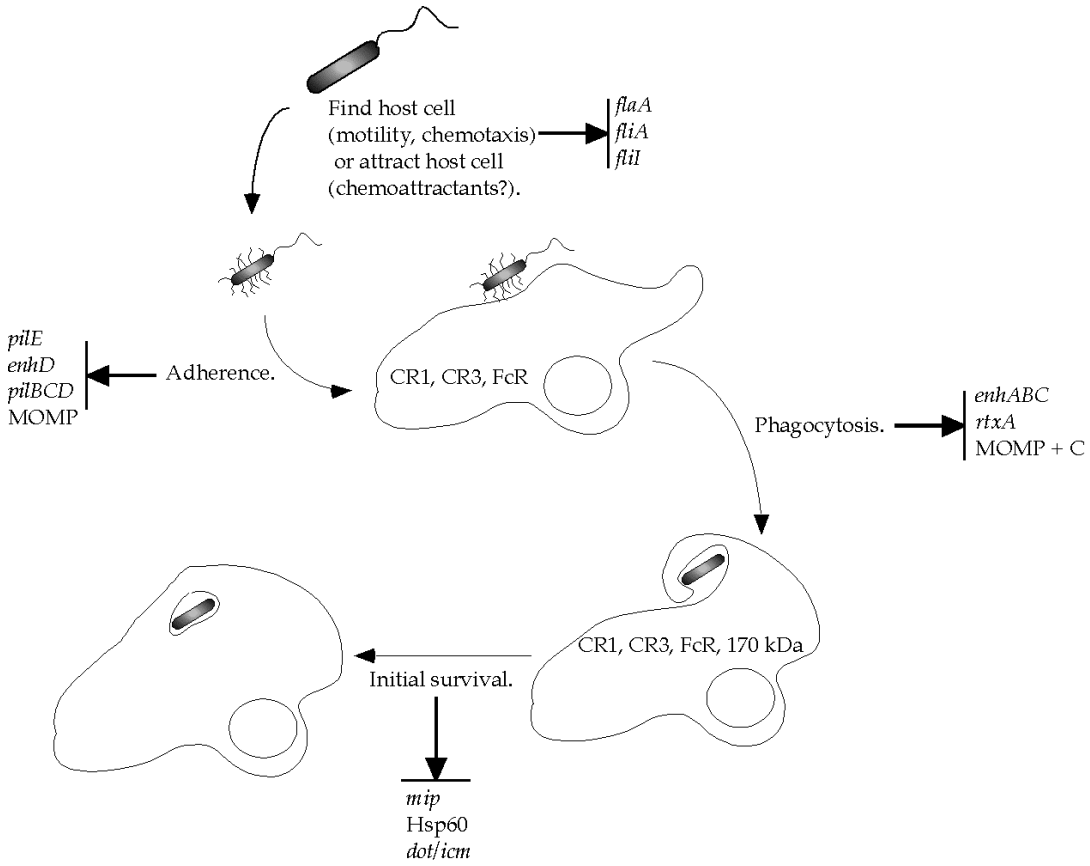

Figure 1. Steps involved in entry into host cells by *Legionella*. The bacteria must first locate the host cells, adhere properly, trigger the appropriate signaling events that lead to phagocytosis and initially survive intracellularly. The bacterial components that are thought to be involved in each of these steps are shown by arrows. Putative host proteins involved are listed within the cell. Abbreviations: MOMP, major outer membrane protein; C, complement; CR1, complement receptor one; CR3, complement receptor three; FcR, Fc receptor; Hsp60, 60 kDa heat-shock protein.

oxidative and other bactericidal mechanisms, inhibition of phagosome-lysosome fusion and interference with phagocyte activation and the subsequent production of bactericidal metabolites (14, 15).

L. pneumophila is an example of an intracellular pathogen that is well adapted to living within a vacuole in phagocytic cells. The *L. pneumophila* vacuole maintains a nearly neutral pH and inhibits fusion with lysosomes (16). Although the molecular basis for inhibition of phagosome-lysosome fusion by *L. pneumophila* is unknown, the mechanism of entry used may play an important role. This possibility is supported by the fact that both *L. pneumophila* (17) and *Toxoplasma gondii* (18) vacuoles will fuse with lysosomes when coated with antibodies prior to infecting host cells expressing Fc receptors. Macrophages possess a number of potential mechanisms that can be used to phagocytose bacterial pathogens. Many of these mechanisms are relatively nonspecific, including LPS- (19), surfactant- (20), Fc- (21-24), complement- (20, 21, 25, 26) and mannose-mediated (27) mechanisms. However, specific mechanisms of entry are also used (28, 29) and may result in a selective advantage during subsequent intracellular events.

Uptake of *L. pneumophila* can occur by an unusual process termed coiling phagocytosis (30-32), where a filipodia from the phagocyte wraps asymmetrically around the bacterium. The only other bacteria that consistently enter monocytes via this mechanism are spirochetes (33-36); whereas, other bacteria almost never (31) or only randomly (37) enter in this manner. Although conventional symmetric phagocytic events are also seen with *L. pneumophila* (31, 38), coiling phagocytosis seems to correlate with intracellular survival and virulence (38). Although *L. pneumophila* are found primarily in monocytes during disease in humans, they can also enter and replicate within epithelial cells (30, 39, 40) and fibroblasts (40, 41). It has been suggested that the interaction of *L. pneumophila* with epithelial cells plays a role in pathogenesis (42). However, direct evidence for a role of host cells other than monocytes in Legionnaires' disease has not been found. The exact molecular mechanism(s) of entry into amoebae, monocytes and epithelial cells and their role(s) in pathogenesis are not well understood.

Entry into host cells by bacterial pathogens, irregardless of the target cell type, involves several steps (figure 1): the ability to find or be found by the target host

cell, adherence, signal transduction, uptake and initial intracellular survival. Thus, entry is a complex multi-factorial process involving a large number of bacterial and host cell components. A review of our current understanding of each step in entry into host cells by *L. pneumophila* may provide insight into the role(s) of the different entry mechanisms that have been observed in Legionnaires' disease.

3. HOW DOES *LEGIONELLA* FIND AN APPROPRIATE HOST CELL?

The first step in entry is the process that brings *L. pneumophila* in close proximity with host cells, allowing intimate interaction. This process may involve motility and chemotaxis by the bacteria or, since the primary host cells, macrophages and amoebae, are motile, the bacteria may secrete factors that attract host cells to them. Although no evidence for the involvement of chemoattractants in entry has been obtained, several investigators have examined factors involved in *L. pneumophila* motility. Shortly after the first identification of *L. pneumophila,* it was demonstrated that the bacteria carry flagella (43) that are 14-20 nm in diameter (44, 45). The flagella are primarily single, subpolar and gently curved but vigorously undulating. However, two or more subpolar flagella are sometimes observed. Characterization of the structural proteins in the flagellum has allowed the identification of the *flaA* gene, which encodes the 47 kDa flagellin subunit (46). Some indication of the importance of the flagellum and, potentially, chemotaxis in pathogenesis by *L. pneumophila* may be gained from examination of regulation of the genes involved.

A number of factors affect the regulation of motility in *L. pneumophila*. Expression of flagella is affected by growth phase (7, 47), temperature (48, 49), nutrients, osmolarity and viscosity (50). The fact that viscosity affects regulation of motility in *L. pneumophila* is consistent with a role in finding host cells within the mucous of mammalian lungs, similar to that observed for *Pseudomonas* (51). Transcription of the flagellar gene is regulated at least in part by an alternative sigma factor $sigma_{28}$, *fliA*. This gene was initially identified by complementation of an *E. coli fliA* mutation (52). It has been suggested that flagella are important for pathogenesis since they have been observed during infection in human lungs (53) and a motile stage occurs during intracellular infection (13). In addition, flagellum expression was correlated with the ability of *L. pneumophila* to grow intracellularly (54) and cause disease (55). However, non-flagellated forms of *L. pneumophila* have been shown to be virulent in guinea pigs and embryonated hens' eggs (56). A mutation in the *L. pneumophila fliI* gene, which affects secretion of flagella, does not reduce the ability to replicate intracellularly in macrophages (57). This study did not; however, examine potential effects on entry into host cells. Subsequently, another group found that a specific *flaA* mutation reduces the ability of *L. pneumophila* to infect both amoebae and macrophages (58). These observations suggest that although motility is not absolutely required for intracellular replication and possibly pathogenesis, it affects the efficiency of the interactions with potential host cells.

Motility may be more important for the growth of *L. pneumophila* in aquatic habitats, where potential host cells are less abundant. In fact, it has been observed that *L. pneumophila* is no longer motile at 24 hours subsequent to release from the intracellular growth environment (59). This observation suggests that, if the bacteria do not find a new potential host cell during this period, they shut down metabolic activity and remain dormant until "eaten" by another protozoan. Clearly, this would not be the preferred outcome for *L. pneumophila*, indicating a strong selective pressure for efficient mechanisms to locate host cells before loss of motility. Currently, chemotaxis toward amoebae and macrophages has not been demonstrated nor have any molecules that attract *L. pneumophila* to these cells been found. In addition, none of the specific isogenic *L. pneumophila* motility mutants have been tested for effects on virulence in animals or competition in a biofilm. Clearly, there is a need for additional research in this area and further evaluation of the potential roles of motility in the ability of *L. pneumophila* to enter host cells.

4. ADHERENCE TO HOST CELLS

Once *L. pneumophila* finds an appropriate host cell that will allow intracellular growth, attachment occurs. Adherence to host cells by bacterial pathogens normally has a direct impact on phagocytosis and potentially on subsequent intracellular events. Thus, it is often difficult to separate adherence from subsequent steps in the entry process. Different methods have been developed to separate adherence from phagocytosis and intracellular survival by inhibiting uptake using temperature (31), formaldehyde fixation (60) or cytochalasin D treatment (61, 62). However, there are problems with nearly all of these techniques that must be taken into consideration. Low temperature incubation of eukaryotic cells reduces adherence of *L. pneumophila,* possibly due to reduced membrane fluidity; formaldehyde fixation may result in improper folding of potential receptors; and cytochalasin D treatment does not fully inhibit uptake of the bacteria (38). Although there remains a need for the development of improved methods for measuring adherence alone, several loci have been identified that are likely to play an important role in this event.

The first putative adherence factor identified for *L. pneumophila* was the major outer membrane protein (MOMP), which is thought to bind complement component C3 (63) and C1q (64). This protein has been recognized as the most abundant *L. pneumophila* outer membrane protein, with a subunit size of approximately 28-29 kDa (65, 66). The MOMP is a porin (67) that is linked to peptidoglycan (67-69) and present in all *Legionella* species (69-71). The primary data indicating that MOMP is involved in *L. pneumophila* attachment are that MOMP-liposomes display enhanced entry into monocytes (63) and that anti-MOMP antibody reduces attachment (61). These studies did not; however, allow determination of whether MOMP-mediated attachment is direct or indirect through complement, since

endogenous complement secreted by macrophages was present in both studies. A strain of *L. pneumophila* with reduced expression of MOMP displays lower attachment and virulence, but this strain was multiply passaged in vitro and uncharacterized genetically (61). Thus, interpretation of the contribution of the MOMP to these observations is unclear. Definitive demonstration of a role for MOMP in attachment awaits the construction and characterization of an isogenic MOMP mutant and complementing strain. In addition, adherence of these strains should be examined in cell lines that do not produce complement, since endogenous complement synthesized by macrophages would make it difficult to determine whether or not complement is required for MOMP-mediated adherence.

Bacterial adhesins can be divided into afimbrial (non-pilus), similar to MOMP, and fimbrial (pilus). The machinery for biogenesis and assembly of pili on the bacterial surface is highly conserved (72). A number of different pili, both curly and straight, were observed on the surface of *L. pneumophila* during very early studies (43, 44). Recent studies resulted in the serendipitous identification of genes, designated *pilB, pilC, pilD* and *pilE*, that are involved in the production and secretion of one of these pili (62, 73). The *pilE* gene encodes a type IV pilus (62) that is secreted by a type II secretory system, which normally requires *pilBCD* (73). These genes appear to be highly similar to the type IV pilus biogenesis locus in *P. aeruginosa* (74): PilB is thought to be involved in assembly of the pilus, PilC is an inner membrane protein that facilitates pilin translocation (75) and PilD is a bifunctional protein that cleaves prepilin and N-methylates the mature pilin subunit (76). Two related loci involved in type II secretion have been identified that affect intracellular replication, but this does not appear to be related to pili production (77, 78). The importance of type IV pili for mediating attachment of several pathogens to epithelial cells has been well documented (75). Similarly, although a *pilE* mutant exhibits wild-type intracellular replication, adherence of the mutant to host cells is significantly reduced (62). This pilus has also been shown to affect natural transformation competence (79) and may be temperature regulated (73).

Although other pili have been observed on the *L. pneumophila* surface (43, 44, 62), their role in adherence has not been examined until recently. A gene, designated *lvhB2*, that encodes a structural pilin subunit protein with similarity to the T-pilus was recently identified as part of the *L. pneumophila* genome project (80). A mutation that deletes *lvhB2* from the *L. pneumophila* chromosome does not affect intracellular replication (80). However, our own group has recently demonstrated that this gene, now designated *enhD* based on its functional classification with other genes that enhance entry, is involved in adherence to monocytes and epithelial cells (81). The *enhD* gene appears to play a role in the efficiency of adherence as well as entry into host cells, but may not directly affect the mechanism by which entry occurs. We are currently exploring whether the role of *enhD* in adherence is also important for the ability of *L. pneumophila* to cause disease. We observed that there are at least two additional types of pili present on the surface of *L. pneumophila* by negative stain, one that is very straight and forms rigid stacks and a second that is relatively short and very thin. The genes that encode these pili have not been identified as yet; thus, it is unclear whether they play a role in adherence. These observations, combined with the possibility that other afimbrial adhesins exist, suggest that we have only begun to understand the complexity of *L. pneumophila* adherence mechanisms.

5. SIGNAL TRANSDUCTION AND UPTAKE

Attachment of *L. pneumophila* to the host cell surface results in signal transduction events that trigger phagocytosis. The details of these signaling events are not well understood. Some of the earliest studies that suggest novel phosphorylation events are involved in the ability of *L. pneumophila* to enter macrophages implicated a 76 kDa protein (82). Although phosphorylation of the 76 kDa protein is *L. pneumophila*-specific, it occurs at 2 hours after infection, suggesting that phosphorylation of this protein is not directly related to entry. A second study examined phosphorylation events in monocytes during earlier time points in detail (83). Although a number of proteins were tyrosine phosphorylated within 15 minutes after infection, all of these proteins were also phosphorylated when the cells were infected with *E. coli*. Tyrosine phosphorylation has been observed in the *L. pneumophila* vacuole in fibroblasts; however, these studies only examined very late time points from two to four hours after entry (84). Thus, it is unclear whether *L. pneumophila* triggers signal transduction events during entry that are specific to virulent bacteria.

Signal transduction has also been examined in protozoa during entry by *L. pneumophila*. Although no phosphorylation events have been observed, a large number of proteins are dephosphorylated in amoebae within 5 to 30 minutes after addition of the bacteria (85). Since several proteins are found to be dephosphorylated in these studies, it is difficult to assess which proteins are directly involved in *L. pneumophila*-host cell interactions. It is also perplexing that dephosphorylation events are thought to be involved in uptake of *L. pneumophila*, since phagocytosis is normally triggered by phosphorylation events. Proteins that are dephosphorylated during entry include a homologue of the 170 kDa Gal/GalNAc lectin from *Entamoeba*, paxillin, $pp125^{FAK}$ and vinculin (85, 86). Although no direct binding of *L. pneumophila* to the 170 kDa lectin has been demonstrated, both galactose and *N*-acetylgalactosamine inhibit uptake into amoebae (85). Since sugar monomers are not specific for the 170 kDa lectin, the inhibition of entry observed in the presence of monoclonal antibodies against this molecule is the most compelling evidence of a role for this receptor in uptake of *L. pneumophila*. However, monoclonal antibodies against the 170 kDa lectin inhibit a maximum of 40% of *L. pneumophila* uptake (85), suggesting that other receptors are likely to be involved. Despite progress in our understanding of these events, there is a pressing need for further examination of signal transduction during entry of *L. pneumophila* into mammalian cells and environmental protozoa.

6. MECHANISMS OF ENTRY

There are several mechanisms that *L. pneumophila* can use to enter host cells. One complicating factor is that *L. pneumophila* can enter several cell types: from epithelial cells and fibroblasts (30, 39-41, 87, 88) to macrophages and amoebae (7, 89). It is unclear whether different mechanisms of entry are used for different cell types or a single method is preferred for all cells. There are likely to be a large number of mechanisms for entry into amoebae and monocytes, where multiple mechanisms exist for non-specific uptake of bacteria as a food source or as part of the host defenses. Early studies in monocytes (90) and polymorphonuclear leukocytes (22) demonstrated that relatively non-specific mechanisms such as opsonization with complement and antibody can increase uptake of *L. pneumophila*. Subsequent studies found that entry by *L. pneumophila* into monocytes occurs by coiling phagocytosis (31). Although this phagocytic event is also commonly observed in spirochetes, the mechanism by which it occurs is not understood (33-36). Opsonization with antibody results in only conventional phagocytic events (31), but does not appear to decrease intracellular viability of *L. pneumophila*. However, antibody in combination with complement greatly increases intracellular killing of the bacteria (90). Opsonization with complement alone does not completely abolish coiling phagocytosis (31), though it can reduce its frequency (38). Bacterial growth conditions can also have an impact on the frequencies of coiling phagocytosis that are observed and prevent a reduction in coiling frequencies by complement opsonization (38). Thus, growth conditions can greatly affect the entry mechanisms used by *L. pneumophila* and should be considered carefully when comparisons are made between studies.

In many of the initial studies that examined *L. pneumophila* entry mechanisms, the bacteria were grown in embryonated hens' eggs (22, 89, 90); whereas, the majority of recent studies use bacteria grown on BCYE agar (91). Growth of *L. pneumophila* intracellularly in amoebae, which may be comparable to growth in hens' eggs, results in nearly 100% coiling phagocytic events as compared to ~40% on BCYE agar (38). Intracellular growth of *L. pneumophila* also dramatically increases the efficiency of entry into host cells (30). A number of differentially expressed membrane proteins were observed in these studies that may be responsible for these observations. However, in contrast to results in hens' eggs (90), entry of amoebae-grown bacteria into monoctyes is not enhanced by complement opsonization (38). It is possible that these differences are strain-specific, but it is equally possible that they are due to differences in the growth conditions used. Direct comparison of the strains and/or growth conditions is necessary in order to differentiate between these possibilities.

Due to the complexity of growth conditions such as embryonated hens' eggs or intracellular growth in amoebae, continued development of improved growth conditions for bacteria to be used for the study of *L. pneumophila* entry mechanisms is ongoing. Phase of growth in liquid media has been suggested as a potentially important factor in expression of some of the genes involved in entry (47). However, although a number of potential virulence factors are regulated by growth phase, *L. pneumophila* grown optimally in liquid media (post-exponential) are less efficient at entering host cells than when grown on standard laboratory media (BCYE) (92). Our laboratory has identified a number of other growth conditions that enhance entry, but none result in entry levels that are comparable to growth intracellularly (J. D. Cirillo, unpublished observations). It appears that regulation of the genes involved in entry by *L. pneumophila* is complex, multifactorial and carefully coordinated to result in expression of the components involved only at the appropriate time, i.e., just prior to release from a host cell.

Complement receptors were found to play a role in entry by *L. pneumophila* into monocytes (25) and these results have been confirmed in subsequent studies (93, 94). However, interactions with complement receptors are most efficient when *L. pneumophila* is also interacting with Fc receptors (94). Possibly, complement receptor interactions occur via binding of MOMP to complement (63) and Fc receptor interactions via anti-lipopolysaccharide antibodies (95). Direct demonstration of these possibilities will require the construction of specific mutations in the bacterial determinants involved. Clearly additional mechanisms of entry exist since opsonin independent entry has been observed in macrophages (38, 96, 97). Furthermore, the other cell types that *L. pneumophila* enters, such as epithelial cells, do not express high levels of complement or Fc receptors. Interestingly, entry into monocytes via non-osponic mechanisms correlates with increased intracellular survival and replication as well as virulence in mice (38). Thus, examination of the mechanisms involved in non-opsonic uptake of *L. pneumophila* is likely to lead to important information regarding pathogenesis.

Recently, we have identified three loci that play a role in non-osponic entry by *L. pneumophila* (98). One of these loci, *enh3*, is only involved in entry into epithelial cells; whereas, the other two, *enh1* and *enh2*, play a role in entry into both monocytes and epithelial cells. Since we have found that growth conditions affecting entry by wild-type *L. pneumophila* into moncoytes almost always affect entry into epithelial cells, we are primarily interested in the *enh1* and *enh2* loci. The *enh1* locus contains a repeats in structural toxin (RTX) gene, designated *rtxA*, and *enh2* contains three genes that encode putative secreted proteins, designated *enhA*, *enhB* and *enhC*. In-frame deletions in the *rtxA* and *enhC* genes reduce entry of *L. pneumophila* into monocytes and epithelial cells by more than 50% (98). Interestingly, the *rtxA* mutation also affects adherence, intracellular replication and virulence in mice (60). It is unclear which of these *rtxA* functions is the most important or whether all of these effects are due to the mechanism of entry and adherence used. It may seem surprising that a toxin would be involved in entry by *L. pneumophila*. However, other RTX proteins have been shown to play a role in adherence and colonization (99). In addition, RTX proteins have recently been shown to bind to β_2 integrins

(100, 101). These observations may at least partially explain the role of complement receptors in entry, since some complement receptors are heterodimeric proteins that contain one β_2 integrin subunit. However, whether *rtxA* in concert with *enhC*, *enhD*, *pilE*, MOMP and/or perhaps other bacterial determinants induces coiling and/or conventional phagocytic mechanisms is unclear. Entry is a complex process in *L. pneumophila* that likely involves both bacterial and host cell components. Recent studies implicating several novel bacterial factors in this process should improve our understanding of entry via the unusual coiling phagocytic mechanism of entry as well as other potential mechanisms.

7. INITIAL INTRACELLULAR SURVIVAL

Although there is usually a lag of 15 to 30 minutes (102, 103), lysosomal fusion may occur during uptake (104). Therefore, it is possible that bacteria are killed by the host cell both during and/or immediately after entry. The *rtxA* gene affects very early intracellular survival as well as adherence and entry (60). Since the impact on intracellular survival is observed very early, within the first 15 minutes, it is likely that this effect is related to entry. The *dot/icm*, *mip* and Hsp60 encoding genes have also been implicated in early events during the intracellular life-cycle of *L. pneumophila*. The *dot*/*icm* system is necessary for proper intracellular trafficking as early as 15 to 30 minutes after entry (105, 106). In addition, *dotH* and *dotO* may play a role in modification of the forming phagosome during entry itself (107). However, surface exposure of *dotH* and *dotO* results in only a limited and transient (10-20 minutes co-incubation) impact on the number of intracellular bacteria, suggesting that their primary role is subsequent rather than prior to the initiation of phagocytosis. However, this does not mean that the *dot*/*icm* complex can not interact with the host cell during both adherence and phagocytosis to some extent. It appears that the activity of the *dot*/*icm* complex is receptor-independent; whereas, earlier events such as adherence and the initiation of phagocytosis are receptor-dependent. Thus, adherence and docking proteins involved in the early aspects of phagoctyosis, such as pili and *rtxA*, primarily affect adherence, entry and the efficiency of *dot*/*icm* complex interactions, but have only a minor role in signaling events that impact trafficking. In contrast, the *dot*/*icm* complex has a relatively minor role in adherence and early stages of entry, but is critical for the final stages of phagocytosis and intracellular trafficking. These conclusions are supported by the recent observations that *dot*/*icm* can trigger a receptor-independent macropinocytic event that appears to reduce the frequency of entry by *L. pneumophila*. Furthermore, in the absence of *dotA L. pneumophila* enters murine macrophages more efficiently, but does not traffick properly. The bacteria used in these studies was strain Lp01-derived and grown under standard laboratory conditions (108). Since adherence and entry is repressed under these growth conditions and Lp01 lacks the entire *lvh* region, including *enhD*, the bacteria used do not express many of the potential adherence and docking proteins involved in the earlier steps of entry by *L. pneumophila*. Thus, *dot*/*icm* complex is required and probably sufficient for proper intracellular trafficking, but efficient entry and optimal trafficking of the bacterial vacuole require other bacterial components. This model suggests that the severity of disease would be impacted by the presence or absence of other adherence and docking proteins, but as long as the *dot*/*icm* complex is present, the bacteria would have the ability to cause disease if a sufficient number of bacteria infect the host.

It has been suggested that Hsp60 is involved in entry into HeLa cells (109). Since these studies examined cell associated and intracellular bacteria after three hours co-incubation, it is unclear whether the effects observed are due to entry or intracellular survival. Two lines of evidence suggest that Hsp60 is important for early intracellular survival. First, several stress proteins, including Hsp60, are induced early during intracellular growth (110-112). Second, these stress-response proteins are often required for optimal intracellular survival and replication (109, 110, 112, 113). Similar results were obtained with *mip*, where mutants in this gene are defective for initial intracellular survival but they have only a slight initial defect in intracellular replication (114, 115). However, the earliest time points examined in these studies were approximately four hours after infection, making it difficult to determine whether *mip* plays a role in early intracellular survival or entry. The absence of an affect on adherence to protozoa (114) suggests that this gene is also involved in intracellular survival rather than entry. Further studies directly investigating affects of genes that are involved in intracellular survival on entry may further clarify their primary biological functions.

8. PERSPECTIVE

This is an exciting time for researchers in the field of *Legionella* pathogenesis. We are just beginning to understand the complexity of the bacterial and host cell interactions involved in entry by *Legionella*. It appears that we have only scratched the surface of possible mechanisms as well as identification of the genes involved. A full understanding of *Legionella* entry mechanisms will require identification of both the bacterial genes and host cell components involved. Construction of specific mutations in putative bacterial entry genes should allow determination of those mechanisms that are preferred by *L. pneumophila*. The effects of opsonization with antibodies and complement on the interaction with host cells (38, 90) suggest that aspects of the entry process are important for subsequent intracellular trafficking and survival. Furthermore, the observation that genes clearly implicated in adherence and entry affect initial intracellular survival supports this conclusion (60). Based on these observations, we have developed a working model for entry of *Legionella* into host cells (figure 2). This model suggests that under ideal growth conditions for *Legionella*, interaction of the bacteria with host cells will primarily trigger the preferred mechanism of entry, initiating intracellular events that favor survival and subsequent

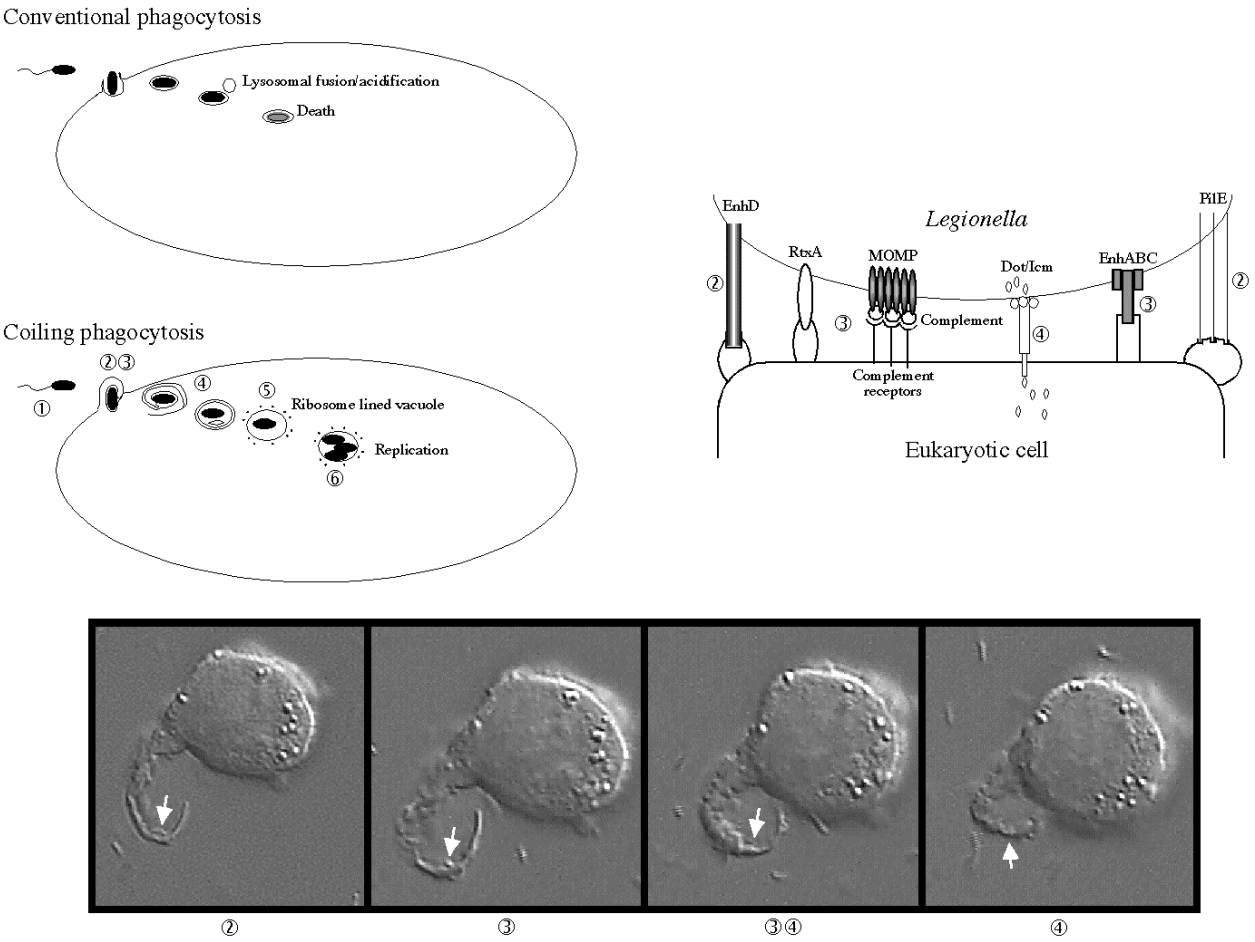

Figure 2. Model for the role of *L. pneumophila* entry mechanisms in intracellular survival and the stages involved in the entry and survival process (1 through 6; top panels). Real-time video microscopy showing entry by *L. pneumophila* into THP-1 monocytic cells by coiling phagocytosis (bottom panels). White arrows indicate the position of *L. pneumophila* in video microscopy panels. Numbers in video microscopy panels correspond to the same stages involved in entry as the cartoon models. Steps 1 through 3 appear to be tightly regulated and often not expressed optimally under standard laboratory growth conditions. Step 1 (chemotaxis and motility) by temperature, nutrient gradients and growth conditions and steps 2 (adherence) and 3 (adherence, docking and possibly signaling) by temperature, growth conditions and iron concentrations. Step 4 (signaling, macropinocytosis and trafficking) appears to be regulated primarily by phase of growth. The preferred mechanism of entry, depicted as coiling phagocytosis, results in optimal or the most efficient signaling and trafficking events leading to intracellular replication, but is probably not absolutely essential for intracellular survival. In contrast, improper entry events, depicted as conventional phagocytosis, result in trafficking events that are detrimental to the survival of *Legionella* but may, at some frequency, be overcome by the activity of the *dot/icm* complex. This model suggests that the most beneficial pathway for interacting with host cells, leading to optimal intracellular survival and replication, is initiated during adherence and signaling events that are associated with intimate attachment and coiling phagocytosis followed by or in concert with the macropinocytic activity of *dot/icm.*

replication. Although uptake events that are not advantageous for *L. pneumophila* are likely to occur at some frequency, due to efforts by the host cell to regain control and destroy the bacteria, it is unclear whether they will always result in bacterial killing. Possibly, *Legionella* has also developed backup strategies that are utilized when the bacteria are exposed to hostile conditions in an intracellular vacuole. It is likely; however, that recovery, modification of and/or escape from a less than optimal intracellular compartment requires a great deal more energy and resources than would be necessary if the bacteria had entered the correct compartment at the outset. Thus, an extended lag period might be expected prior to the initiation of intracellular replication if a suboptimal entry mechanism is used by the majority of bacteria entering. Our ability to understand these important events in *L. pneumophila* pathogenesis will be greatly enhanced with a more comprehensive understanding of the bacterial and host cell components involved. We expect that the upcoming determination of the complete *L. pneumophila* genome sequence will increase the pace at which the process of identifying these genes can proceed.

9. ACKNOWLEDGMENTS

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